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Note

Prepurification and derivatization of α-keto acids using hydrazide gel

Application in gas chromatography and gas chromatography—mass spectrometry

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Several methods [1—8] have been reported for the analysis of α-keto acids by means of gas chromatography and/or gas chromatography—mass spectrometry using certain kinds of derivatization. The methods most widely employed have been those using 2,4-dinitrophenylhydrazine [9, 10]. However, with these methods chromatographic separations were complicated since the derivatives of α-keto acids exist as *syn—anti* isomers. Moreover, no effective clean-up methods have been reported. In a previous paper [11] we reported that the clean-up and derivatization of α-keto acids using hydrazide gel was very effective for the high-performance liquid chromatographic method. This present paper describes the application of this pretreatment to the gas chromatographic analysis of α-keto acids in biological samples.

MATERIALS AND METHODS

Apparatus

The gas chromatograph (Model 163, Hitachi, Tokyo, Japan) used was equipped with a flame ionization detector. The separation was carried out using a Dexsil 300GC capillary column (20 m × 0.25 mm I.D., support-coated,

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open-tube capillary column; Gasukuro Kogyo Co., Tokyo, Japan), of which the split ratio was 1:30. Chromatography was conducted at an initial temperature of 160°C with temperature programming at the rate of 2°C/min to a temperature of about 250°C, then isothermally at 310°C for 10 min. The injection port temperature was 320°C and the detector temperature was 300°C. The nitrogen carrier gas flow-rate was 1.2 ml/min. Gas chromatographic—mass spectrometric measurement was performed on a Hitachi Model M-80/M-003 using an electron-accelerating energy of 20 eV. Conditions for gas chromatography were as described above, with the following exceptions: the carrier gas (helium) flow-rate was 1.0 ml/min, and the temperature program was 5°C/min (160—260°C).

Reagents

Sodium pyruvate (PA), sodium α-ketoisovalerate (KIVA), sodium α-ketoisocaproate (KICA), sodium α-keto-β-methylvalerate (KMVA), sodium α-ketobutyrate (KBA), sodium α-ketovalerate (KVA), and phenylpyruvic acid (PPA) were obtained from Sigma (St. Louis, MO, U.S.A.). *o*-Phenylenediamine sulfate was purchased from Tokyo Chemical Industry Co. (Tokyo, Japan) and used after recrystallization from a mixture of 1% aqueous sulfuric acid and ethanol (1:1). Bio-gel P-60 (100—200 mesh) was obtained from Bio-Rad Labs. (Richmond, CA, U.S.A.). Pyridine and N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Wako Pure Chemicals (Osaka, Japan). The other reagents and solvents were reagent grade.

The preparation of hydrazide gel was as follows. Dry polyacrylamide beads (15 g) were allowed to swell overnight in distilled water (200 ml) contained in a siliconized, conical glass-stoppered flask. The flask with the gel suspension and a glass-stoppered cylinder containing 98% hydrazine hydrate (120 ml) were immersed in a constant-temperature water bath (50°C). After 45 min the hydrazine hydrate was added to the gel, the flask was stoppered, and the mixture was stirred by an immersible magnetic stirrer for 6 h. At the end of the reaction period, the gel was washed with 0.1 *M* NaCl on a Buchner funnel until the washings were essentially free of hydrazine. The gel was stored under refrigeration suspended in an approximately pH 7.3 buffer of the following composition: 0.20 *M* NaCl, 0.02 *M* disodium EDTA, 0.10 *M* H_3BO_3, 0.005 *M* NaOH and $5 \cdot 10^{-6}$ *M* pentachlorophenol. The specific capacity of the gel was measured in the following manner. A certain volume of the gel was resuspended in 0.1 *M* NaCl, and a certain excess of pyruvate was added to the suspension and the solution was made moderately acidic with 0.1 *M* acetic acid. Then the gel was filtered and washed with 0.1 *M* NaCl. The filtrates and washings were combined. The amount of pyruvate in this solution was determined using the high-performance liquid chromatographic method. Next, pyruvate trapped in the gel was estimated.

Reagent preparation

All aqueous reagent solutions were prepared with redistilled water. The standard stock solutions of α-keto acids were prepared separately at the concentrations of 2 μmol/ml in water or 10% aqueous ethanol (PPA). A standard mixture of α-keto acids was prepared by mixing the standard stock solutions

and diluting with redistilled water to contain 400 nmol of each α-keto acid per milliliter. *o*-Phenylenediamine solution was prepared by dissolving 40 mg of *o*-phenylenediamine sulfate in 40 ml of 2 *N* HCl.

Prepurification and derivatization of α-keto acids in human urine

Three milliliters of 0.2 *M* aqueous acetic acid and 6 ml of 0.1 *M* NaCl solution were added to 3 ml of human urine. The mixture was poured into a siliconized glass column (150 mm × 8 mm I.D.) containing 1 ml of hydrazide gel. After the column was drained, the gel was washed with 20 ml of 0.1 *M* NaCl solution. The gel was then transferred to a test tube. Four milliliters of *o*-phenylenediamine solution were added to it and the mixture was warmed in a water bath at about 80°C for 2 h. At the end of the reaction period, the reaction mixture was diluted with 16 ml of a saturated sodium sulfate solution. The products were extracted into 20 ml of ethyl acetate by shaking for 5 min. The organic phase was dried over anhydrous sodium sulfate and evaporated to dryness. The residue was redissolved in 10 μl of pyridine and reacted with 50 μl of BSTFA. About 0.1 μl of the reaction mixture was injected into the gas chromatograph.

Prepurification and derivatization of α-keto acids in human plasma

Ten milliliters of methanol were added to 3 ml of human plasma and shaken vigorously and centrifuged. The methanol in the supernatant was evaporated on a rotary evaporator. Three milliliters of 0.2 *M* acetic acid solution and 6 ml of 0.1 *M* NaCl solution were added to the residual solution and mixed. The solution was then treated in the same manner as with the human urine sample.

RESULTS AND DISCUSSION

Figs. 1 and 2 show a typical chromatogram and mass spectra, respectively, as obtained from a standard mixture of TMS-quinoxalinols derived from α-keto acids. The good separation, especially in the case of the derivatives of KIVA, KMVA and KICA, was the result of using a glass capillary column instead of a packed column. The mass spectra in Fig. 2 were measured at the top of each peak and corrected by the background.

Fig. 3A shows the gas chromatogram of a normal human urine sample. The components which gave the same retentions as those of PA, KMVA and KICA derivatives were identified by comparing each mass spectrum with that of the corresponding authentic derivatives. The gas chromatogram shown in Fig. 3B is from a urine sample of a phenylketonuria (PKU) patient. The peak components of corresponding to PA and PPA were also identified by comparing the mass spectra.

Fig. 4 shows chromatograms of plasma samples from a normal human and from a patient with maple syrup urine disease (MSUD). The gas chromatogram of plasma from the MSUD patient was dominated by the large peaks of KMVA, KIVA and KICA derivatives. Virtually no peaks were found on the chromatograms except those from α-keto acids. The data mentioned above seem to indicate that our prepurification and derivatization were very effective for gas chromatography of α-keto acids in biological samples.

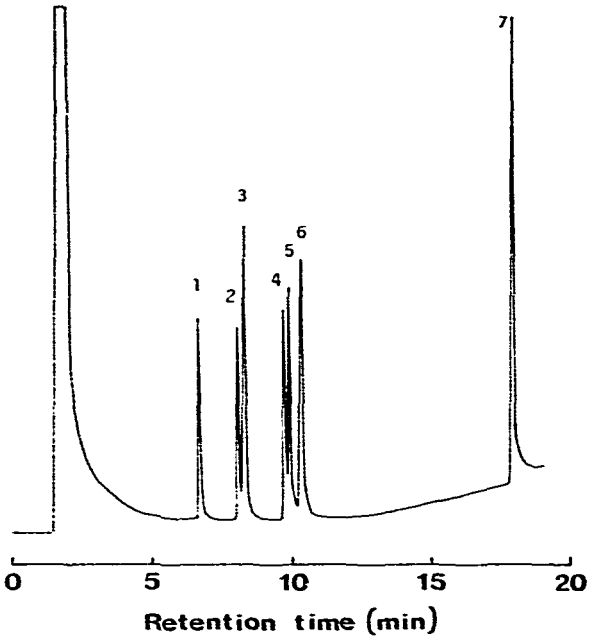

Fig. 1. Gas chromatogram of standard mixture of the TMS-quinoxalinol derivatives of the α-keto acids. Peaks: 1 = PA, 2 = KBA, 3 = KIVA, 4 = KVA, 5 = KMVA, 6 = KICA, 7 = PPA.

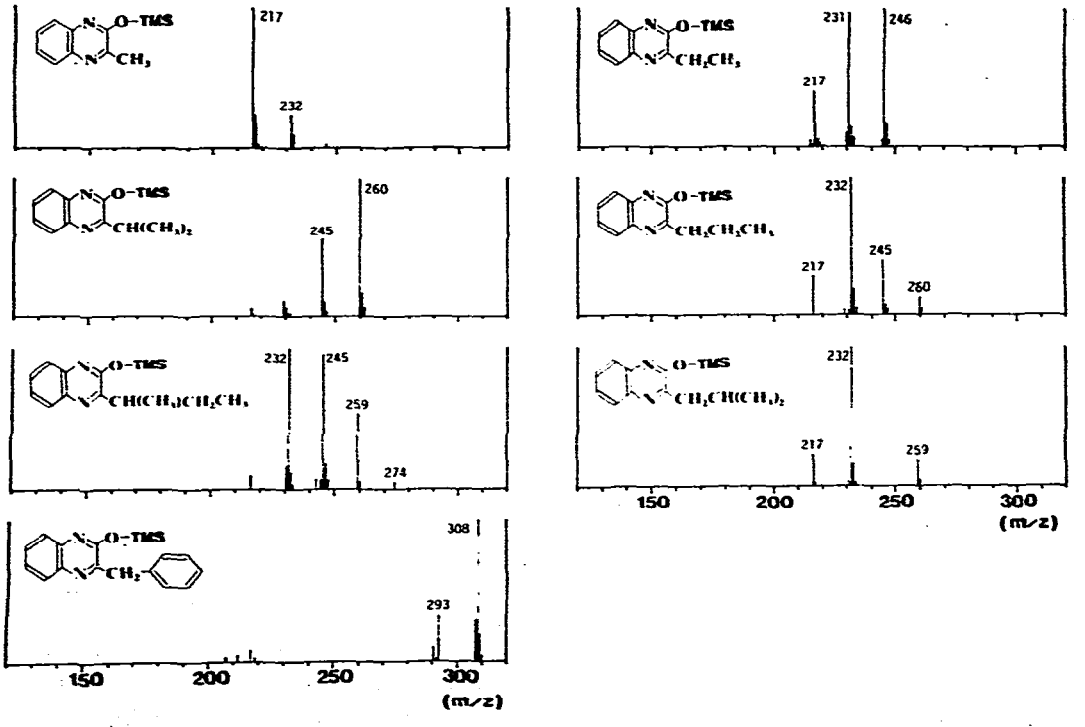

Fig. 2. Mass spectra of TMS-quinoxalinol derivatives of α-keto acids.

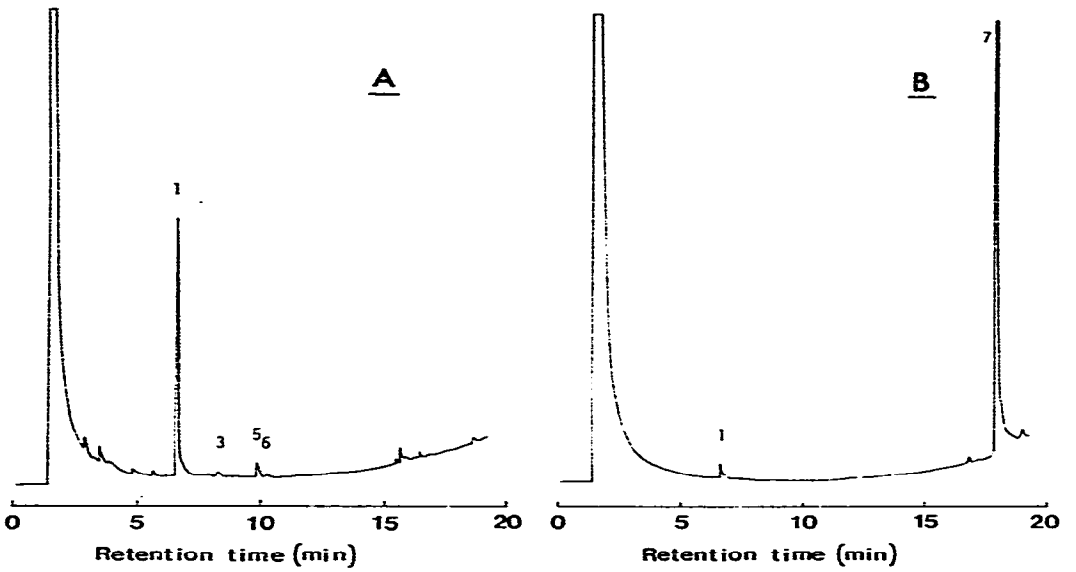

Fig. 3. Gas chromatograms obtained from urine samples of a normal subject (A), and of a PKU patient (B).

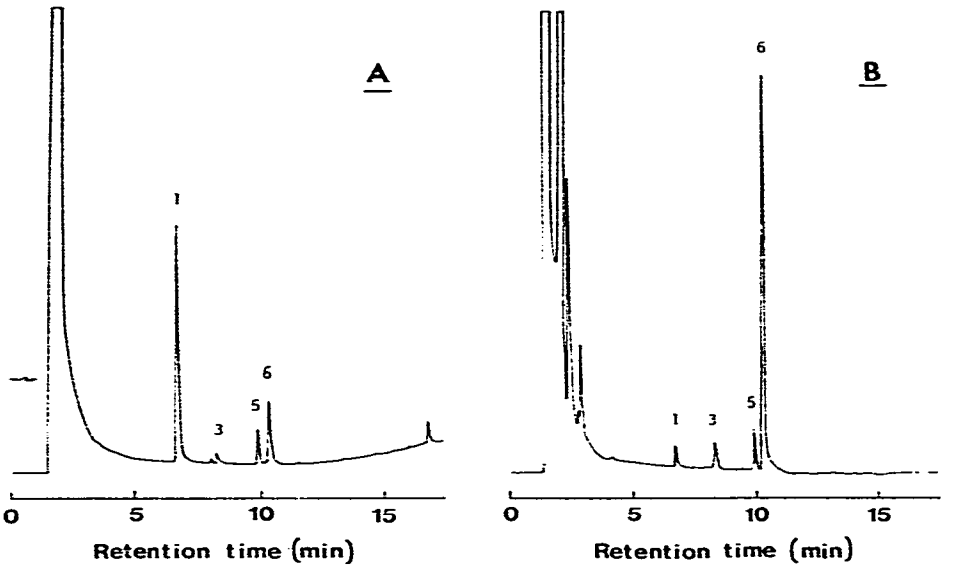

Fig. 4. Gas chromatograms obtained from plasma samples of a normal subject (A), and of a MSUD patient (B).

Most reported methods use extraction with some kind of organic solvent for prepurification and condensation after the reaction with reagent in an aqueous medium. Cree et al. [6] prepurified the branched-chain α-keto acids from physiological sources by deproteinization with acetone, and cation-exchange chromatography. However, these methods seem to be time consuming and not very effective.

The new prepurification and derivatization method which was confirmed to be very useful for the high-performance liquid chromatographic determination of α-keto acids in biological samples was also effective as a method of pretreatment for gas chromatography.

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REFERENCES

1 H. Sternowsky, J. Roboz, F. Hutter and G. Gaull, Clin. Chim. Acta, 47 (1977) 371.
2 U. Langenbeck, H.-U. Möhring and K.-P. Dieckmann, J. Chromatogr., 115 (1975) 65.
3 U. Langenbeck, A. Hoinowsky, K. Mantel and H.-U. Möhring, J. Chromatogr., 143 (1977) 39.
4 U. Langenbeck, H.-U. Möhring, B. Hinney and M. Spiteller, Biomed. Mass Spectrom., 4 (1977) 197.
5 G.C. Hemming and C.J. Gubler, Anal. Biochem., 92 (1979) 31.
6 T.C. Cree, S.M. Hutson and A.E. Harper, Anal. Biochem., 92 (1979) 156.
7 H. Nakamura and Z. Tamura, Anal. Chem., 51 (1979) 1679.
8 S. Ohmori, Y. Tanaka, M. Ikeda and K. Hirota, Anal. Biochem., 112 (1981) 204.
9 H. Kallio and R.R. Linko, J. Chromatogr., 76 (1973) 229.
10 P. Ronkainen and S. Brummer, J. Chromatogr., 28 (1967) 259.
11 T. Hayashi, H. Tsuchiya, H. Todoriki and H. Naruse, Anal. Biochem., 122 (1982) 173.